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Inorganic Polyphosphate Is Required for Motility of Bacterial Pathogens

M. HARUNUR RASHID, NARAYANA N. RAO, AND ARTHUR KORNBERG*

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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The *ppk* gene encodes polyphosphate kinase (PPK), the principal enzyme in many bacteria responsible for the synthesis of inorganic polyphosphate (polyP) from ATP. A null mutation in the *ppk* gene of six bacterial pathogens renders them greatly impaired in motility on semisolid agar plates; this defect can be corrected by the introduction of *ppk* gene in *trans*. In view of the fact that the motility of pathogens is essential to invade and establish systemic infections in host cells, this impairment in motility suggests a crucial and essential role of PPK or polyP in bacterial pathogenesis.

Inorganic polyphosphate (polyP) is a linear polymer of hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds. It is ubiquitous in nature (10), having been found in every bacterial, plant, and animal cell. Among the many functions proposed for polyP is a role in *Escherichia coli* in regulating the networks essential for responses to nutritional stringencies and environmental stresses and for survival in the stationary phase of growth (2, 15, 16). The regulatory role in *E. coli* is inferred from the behavior of null mutants of *ppk*, the gene that encodes polyP kinase (PPK), the enzyme responsible for the synthesis of polyP from ATP.

Inasmuch as some virulence factors are also expressed in stationary phase (11, 19), there may be a dependence on polyP for gene regulation in a pathogen (23). The relationship of polyP to virulence in pathogens is suggested by three observations: (i) massive accumulation of polyP in *Helicobacter pylori* during its infectious stage (S. Liu and A. Kornberg, unpublished data), (ii) sensitivity of the *ppk* mutant of *Neisseria meningitidis* to 10% human serum (22), and (iii) coregulation of capsular polysaccharide (alginate) synthesis and polyP accumulation in *Pseudomonas aeruginosa* (9).

Among these three pathogens and at least ten others, there is a remarkable conservation of the PPK amino acid sequence (reference 23 and C.-M. Tzeng and A. Kornberg, unpublished data). This list of pathogens includes *P. aeruginosa*, which causes infections in humans, particularly with cystic fibrosis patients, burn victims, and individuals with AIDS or cancer. Other pathogens in this list are *Salmonella* spp., the causative agents of typhoid and/or gastroenteritis; *Vibrio cholerae*, the cause of severe diarrheal disease; *Klebsiella pneumoniae*, an agent of pneumonia in humans; *H. pylori*, the cause of chronic gastritis and peptic ulcers; and *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the agents of tuberculosis and leprosy, respectively.

To elucidate the roles of polyP in bacterial pathogenesis, we prepared *ppk* null mutants of *P. aeruginosa* PAO1, *V. cholerae* 92A1552, *Salmonella enterica* serovar Typhimurium FIRN, and *Salmonella enterica* serovar Dublin SVA47, in addition to the available *E. coli* MG1655 and *K. pneumoniae* ATCC9621 mutants. The *ppk::tet* mutation in *P. aeruginosa* and the *ppk::kan* mutations in *V. cholerae* and *Salmonella* serovars Typhimurium and Dublin were verified at the genetic level either by genomic PCR or Southern hybridization. Biochemical verifications were performed by assaying the loss of activities of PPK and exopolyphosphatase (PPX) (where appropriate) relative to the wild type and by the deficiency in polyP accumulation under defined conditions (unpublished data).

To examine whether the *ppk* mutation has any effect on the flagellar motility of these pathogens, the motility of these mutants was compared with that of the corresponding wild-type strains on swim plates (1% tryptone, 0.5% NaCl) containing 0.3% agar. As shown in Fig. 1 for *P. aeruginosa*, the mutant is severely impaired in motility. Since the *P. aeruginosa ppk* mutant still accumulates at least 20% as much polyP under some conditions compared to the wild type (unpublished data), the mutant was transformed with a plasmid overexpressing the yeast PPX (ScPPX1) (24) to deplete residual polyP. This strain behaved much like the mutant. When the mutant was transformed with a plasmid expressing *P. aeruginosa* PPK, the motility was completely restored. This clearly demonstrates the dependence of flagellar motility on PPK function. This observation has been extended to other pathogens (Table 1). Impairments of swarming in the *ppk* mutants were between 13 and 79% of those of the wild-type levels. As in *P. aeruginosa*, the motility deficiency of the mutant could be complemented in *E. coli* by introducing the *ppk* gene on a plasmid.

To determine whether the impairment in swimming motility of the *ppk* mutants on semisolid agar plates is due to a growth impairment, the growth of *E. coli* and *P. aeruginosa* wild-type and *ppk* mutants was monitored in shaking cultures at 30°C in tryptone broth. No growth defect could be observed (Fig. 2) to account for the reduced swimming motility of the *ppk* mutants on semisolid tryptone plates. Electron microscopy revealed that the *ppk* mutants of *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *V. cholerae*, and *Salmonella* serovar Dublin all possessed apparently intact flagella indistinguishable from those of the wild-type strains (data not shown). Thus, the effect of polyP on swimming motility is likely due to altered functioning of the flagella.

Direct microscopic observations revealed that the *E. coli* and *P. aeruginosa ppk* mutants were motile in liquid culture. Cells were in exponential phase (0.4 to 0.7 optical density at 600 nm) grown in tryptone broth (1% tryptone, 0.5% NaCl) at 30°C. Peritrichous *E. coli* and monotrichous *P. aeruginosa* change direction of movement by similar mechanisms, a reversal of flagellar rotation (21); in *E. coli*, reversal causes tumbling, and in *P. aeruginosa*, reversal causes the bacteria to back up. The

* Corresponding author. Mailing address: Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305. Phone: (650) 723-6167. Fax: (650) 723-6783. E-mail: akornber@cmgm.stanford.edu.

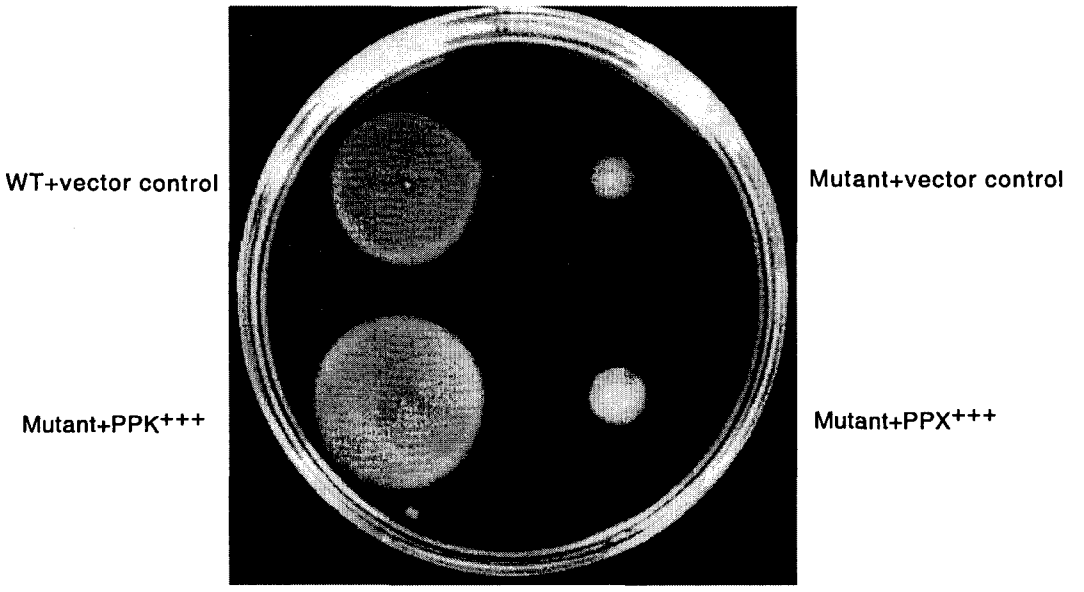

FIG. 1. Swimming motility of *P. aeruginosa* PAO1 wild-type (WT) and derivative strains. The flagellum-mediated motility of the strains was assessed on tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) with carbenicillin (300 μg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) after 12 h of growth at 30°C. Migration of the cells from the point of inoculation (observed as a turbid zone) indicates that a strain is proficient for flagellar-mediated motility. The strains are (clockwise from upper left) PAO1/p66HE (WT plus vector control), PAOM-5/p66HE (Δ*ppk* plus vector control), PAOM-5/pSCPPX (Δ*ppk* plus PPX^{+++}), and PAOM-5/pPAPPK (Δ*ppk* plus PPK^{+++}).

ppk mutants of *E. coli* and *P. aeruginosa* along with their respective wild-types examined under phase-contrast microscopy (magnification, ×800) revealed no striking differences in changes of movement direction between wild-type and mutant cells. Possibly, more refined techniques will disclose the role of polyP in flagellar swimming.

Flagella are highly complex and conserved bacterial organelles requiring coordinated and ordered expression of about 50 genes for their synthesis and function (18). The roles of flagella in chemotaxis and motility are important for the survival of many organisms. A connection between virulence and flagellum-based motility has long been observed in many pathogens, some of which require functional flagella for virulence (7, 8, 12) and others in which motility must be suppressed for virulence (1).

The roles of flagella and flagellum-mediated motility in *P. aeruginosa* pulmonary and burn infections have been studied in detail (4–6, 13, 20). In the pathogenesis of respiratory tract infection, it has been shown that flagella and/or flagellar motility are necessary at three distinct stages of infection: (i) acquisition of motile organisms, (ii) immunostimulation, and (iii) adaptation (6). Flagellar motility and type IV pili-based twitching motility have been found necessary for the development of a *P. aeruginosa* biofilm (14). Bacterial biofilms are troublesome when they form on tissues, on catheters, or on medical implants because of their innate resistance to antibiotics and

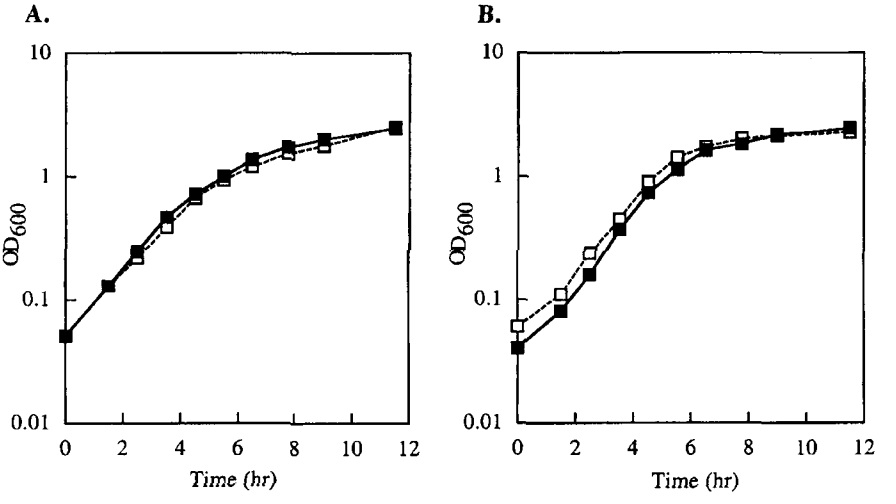

FIG. 2. Growth curves of *E. coli* MG1655 (A) and *P. aeruginosa* PAO1 (B) wild-type and *ppk* mutants in tryptone broth at 30°C. Growth was monitored at an optical density at 600 nm (OD_{600}). Symbols: ■, wild type; □, mutant (*ppk*).

TABLE 1. Flagellum-mediated motility of pathogens on swim plates

Strain	Relevant genotype[a]	Swim area (% WT ± SEM)[b]
E. coli MG1655	WT	100 ± 7.0
	Δ*ppk-ppx*	46 ± 3.5
	WT + vector	100 ± 7.3
	Δ*ppk-ppx* + vector	33 ± 4.7
	Δ*ppk-ppx* + *ppk*$^{+++}$	91 ± 6.6
P. aeruginosa PAO1	WT	100 ± 12.7
	Δ*ppk*	31 ± 1.8
	WT + vector	100 ± 8.9
	Δ*ppk* + vector	13 ± 1.7
	Δ*ppk* + *ppx*$^{+++}$	13 ± 1.2
	Δ*ppk* + *ppk*$^{+++}$	92 ± 14.7
K. pneumoniae ATCC9621	WT	100 ± 5.0
	Δ*ppk-ppx*	33 ± 0.7
V. cholerae 92A1552	WT	100 ± 4.5
	Δ*ppk*	57 ± 4.8
Salmonella serovar Dublin SVA47	WT	100 ± 3.6
	Δ*ppk-ppx*	58 ± 3.8
Salmonella serovar Typhimurium FIRN	WT	100 ± 6.4
	Δ*ppk-ppx*	79 ± 8.6

[a] WT, wild type.

[b] Swim area was measured after 10 to 12 h of incubation at 37°C on tryptone swim plates. The standard error of the mean equals $\sigma_{n-1}/\sqrt{n}$ (17), where $n = 10$, except for *P. aeruginosa*, where incubation was at 30°C and $n = 4$. WT, wild type.

other biocides (for a review, see reference 3). We have found that the *ppk* mutant of *P. aeruginosa* is also defective in twitching motility and biofilm formation on abiotic surfaces (data not shown). Taken together, these several lines of evidence suggest that PPK or polyP might be a virulence determinant of pathogens, like *P. aeruginosa*.

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